

Isoform-selective NADPH oxidase inhibitor panel for pharmacological target validation

Citation for published version (APA):

Vu Thao-Vi Dao, Elbatreek, M. H., Altenhofer, S., Casas, A., Pachado, M. P., Neullens, C. T., Knaus, U. G., & Schmidt, H. H. H. W. (2020). Isoform-selective NADPH oxidase inhibitor panel for pharmacological target validation. *Free Radical Biology and Medicine*, 148, 60-69.
<https://doi.org/10.1016/j.freeradbiomed.2019.12.038>

Document status and date:

Published: 20/02/2020

DOI:

[10.1016/j.freeradbiomed.2019.12.038](https://doi.org/10.1016/j.freeradbiomed.2019.12.038)

Document Version:

Publisher's PDF, also known as Version of record

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NOX Inhibitors: From Bench to Naxibs to Bedside

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and Harald H. H. W. Schmidt

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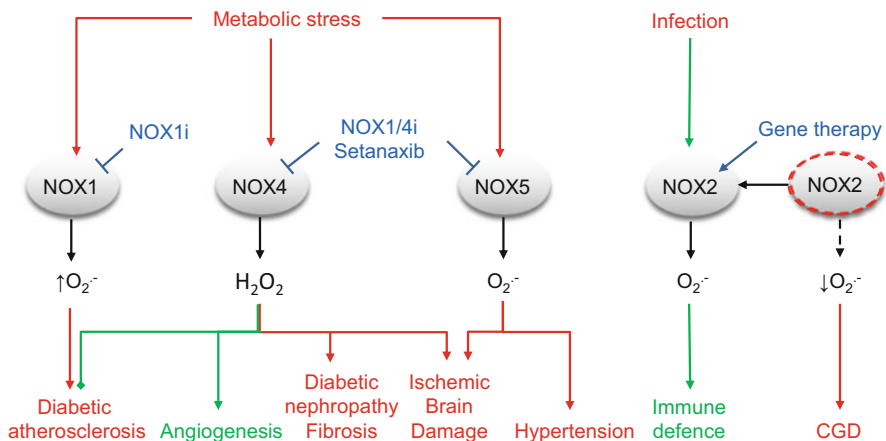
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Handbook of Experimental Pharmacology, https://doi.org/10.1007/164_2020_387

Abstract

Reactive oxygen species (ROS) are ubiquitous metabolic products and important cellular signaling molecules that contribute to several biological functions. Pathophysiology arises when ROS are generated either in excess or in cell types or subcellular locations that normally do not produce ROS or when non-physiological types of ROS (e.g., superoxide instead of hydrogen peroxide) are formed. In the latter scenario, antioxidants were considered as the apparent remedy but, clinically, have consistently failed and even sometimes induced harm. The obvious reason for that is the non-selective ROS scavenging effects of antioxidants which interfere with both qualities of ROS, physiological and pathological. Therefore, it is essential to overcome this “antidote or neutralizer” strategy. We here review the most promising alternative approach by identifying the disease-relevant enzymatic sources of ROS, target these selectively, but leave physiological ROS signaling through other sources intact. Among all ROS sources, NADPH oxidases (NOX1-5 and DUOX1-2) stand out as their sole function is to produce ROS, whereas most other enzymatic sources only produce ROS as a by-product or upon biochemical uncoupling or damage. This qualifies NOXs as the main potential drug-target candidates in diseases associated with dysfunction in ROS signaling. As a reflection of this, the development of several NOX inhibitors has taken place. Recently, the WHO approved a new stem, “naxib,” which refers to NADPH oxidase inhibitors, and thereby recognized NOX inhibitors as a new therapeutic class. This has been announced while clinical trials with the first-in-class compound, setanaxib (initially known as GKT137831) had been initiated. We also review the differences between the seven NOX family members in terms of structure and function in health and disease and then focus on the most advanced NOX inhibitors with an exclusive focus on clinically relevant validations and applications.

Graphical Abstract



Therapeutically relevant NADPH oxidase isoforms type 1, 2, 4, and 5 (NOX1, NOX2, NOX4, NOX5). Of note, NOX5 is not present in mice and rats and thus pre-clinically less studied. NOX2, formerly termed gp91^{phox}, has been correlated with many, too many, diseases and is rather relevant as genetic deficiency in chronic granulomatous disease (CGD), treated by gene therapy. Overproduction of ROS through NOX1, NOX4, and NOX5 leads to the indicated diseases states including atherosclerosis (red), a condition where NOX4 is surprisingly protective.

Keywords

Mechanism-based redox therapeutics · NADPH oxidases · NOX inhibitors · Setanaxib · Reactive oxygen species

1 The NADPH Oxidase Family of Enzymes

NADPH oxidases (NOXs) are transmembrane enzymes that transfer electrons from NADPH in the cytoplasm across the cell membrane resulting in the formation of reactive oxygen species (ROS) (Cross and Segal 2004; Panday et al. 2015). The NOX family consists of seven members, NOX1–5 and the dual oxidases DUOX1–2. These enzymes are different in terms of enzyme complex composition, tissue and cellular distributions, subcellular localizations, mechanisms of activation, and the ROS type they produce. Thus, they are implicated in diverse physiological functions and disease conditions (Altenhofer et al. 2015; Casas et al. 2015; Elbatreek et al. 2019).

NOX2 (formerly known as gp91^{phox}) was the first NOX family member to be discovered (Rossi and Zatti 1964; Segal and Jones 1978). Other NOXs were discovered later and share certain sequence homology with this isoform, 56% for NOX1 (aka Mox1), 58% for NOX3 (aka MOX2), 39% for NOX4 (aka Renox), 27% for NOX5, 57% for DUOX1 (aka ThOX1), and 43% for DUOX2 (aka ThOX2) (Cheng et al. 2001; De Deken et al. 2000; Suh et al. 1999). With respect to structure, all the family members possess a catalytic subunit, NOX, which is formed of six- or seven-transmembrane helices in NOX1–5 and DUOX1–2, respectively. NOX subunit binds two heme cofactors and allows for NADPH oxidation through a FAD/NADPH-binding domain in the cytosolic C-terminus (Cheng et al. 2001; Meitzler and Ortiz de Montellano 2009, 2011). In the case of NOX5 and DUOX1–2, NOX also binds to an intracellular Ca²⁺-binding EF-hand region (Banfi et al. 2001, 2004b; Rigutto et al. 2009). Besides, NOXs differ in their membrane or cytosolic binding partners that are required for the enzymatic activity.

In NOX1–4, a membrane-bound subunit, p22^{phox}, is required for stabilization, whereas DUOX1 and 2 associate with the membrane maturation factors DUOXA1 and 2, respectively (Ambasta et al. 2004; Grasberger and Refetoff 2006; Parkos et al. 1987; Ueno et al. 2005). NOXs are also associated with cytosolic activator proteins (NOXA1 for NOX1 and NOX3, p67^{phox} and p40^{phox} for NOX2) which increase

enzymatic ROS-forming activity and organizer proteins (NOXO1 for NOX1 and NOX3, p47^{phox} for NOX2) that help tether the activators with the NOX subunit (Banfi et al. 2003, 2004a; Volpp et al. 1988; Wientjes et al. 1993). In addition, other binding proteins help regulate NOX activity such as the small GTPase, RAC1, for NOX1–3 (Cheng et al. 2006; Diebold and Bokoch 2001; Ueyama et al. 2006), polymerase δ -interacting protein 2 (POLDIP2) for NOX4 (Lyle et al. 2009), and heat shock protein 90 (HSP90) for NOX1–3 and NOX5 (Chen et al. 2011).

Regarding their tissue and cellular distribution, the seven NOXs are widely expressed throughout different tissues (Fig. 1). NOX1 is predominantly expressed in colon epithelium (Szanto et al. 2005) and also in the uterus (Banfi et al. 2000; Suh et al. 1999), placenta (Cui et al. 2006), prostate (Banfi et al. 2000; Suh et al. 1999), pancreas (Xia et al. 2019), retina (Manea et al. 2005), keratinocytes (Chamulitrat et al. 2003), endothelium (Gray et al. 2013), and vascular smooth muscle cells (Lassegue et al. 2001). NOX2 is expressed in phagocytes which are present in numerous tissues and is often called “the phagocyte NADPH oxidase” (Bedard and Krause 2007); however, it can also be detected in other cell types including cardiomyocytes (Krijnen et al. 2003), skeletal muscle (Henriquez-Olguin et al. 2019), endothelial cells (Gorlach et al. 2000), hepatocytes (Reinehr et al. 2005), and neurons (Fan et al. 2019). NOX3 is highly abundant in the inner ear (Banfi et al. 2004a) in addition to other fetal tissues (Banfi et al. 2004a; Cheng et al. 2001), while NOX4 is highly expressed in kidney cells (Geiszt et al. 2000; Gorin et al. 2003; Jha et al. 2016), endothelium (Van Buul et al. 2005), vascular smooth muscle cells (Hoidal et al. 2003), cardiomyocytes (Brewer et al. 2011), fibroblasts (Cucoranu et al. 2005), adipocytes (Den Hartigh et al. 2017), and neurons (Casas et al. 2017). NOX5, which is absent in rodents, shows substantial expression in the testis, spleen, and lymph nodes (Banfi et al. 2001) and is also detected in the endothelial cells (BelAiba et al. 2007), vascular smooth muscle cells (Jay et al. 2008), kidney (Holterman et al. 2014; Jha et al. 2017a), and white blood cells (Manea et al. 2015). DUOX1–2 are predominantly found in the thyroid gland (De Deken et al. 2000) in addition to the lung epithelia (Fischer 2009) and prostate (D. Wang et al. 2005). DUOX1 is also expressed in epidermal keratinocytes (Ko et al. 2014) and DUOX2 in salivary ducts and the gastrointestinal tract (El Hassani et al. 2005; Geiszt et al. 2003b).

The subcellular localization/compartmentalization varies between NOXs in different cell types; however, the data are limited by the lack of high-quality antibodies against these enzymes (Zhang et al. 2019). NOX1 is localized in the endoplasmic reticulum, caveolae, and nucleus (Chamulitrat et al. 2003; Hilenski et al. 2004; Janiszewski et al. 2005), while NOX2 is present at the plasma membrane, perinuclear cytoskeleton, and endoplasmic reticulum (Huang et al. 1995; Krijnen et al. 2003; Segal and Jones 1978; Van Buul et al. 2005). There is barely any information about the subcellular localization of NOX3; however, one study showed the co-localization of NOX3 and p22^{phox} in the plasma membrane of transfected HEK-293 cells (Nakano et al. 2007). NOX4 and NOX5 are localized at the cell membrane, nucleus, endoplasmic reticulum, and mitochondria (Ago et al. 2010; BelAiba et al. 2007; Case et al. 2013; Hilenski et al. 2004; Marzaioli et al. 2017;

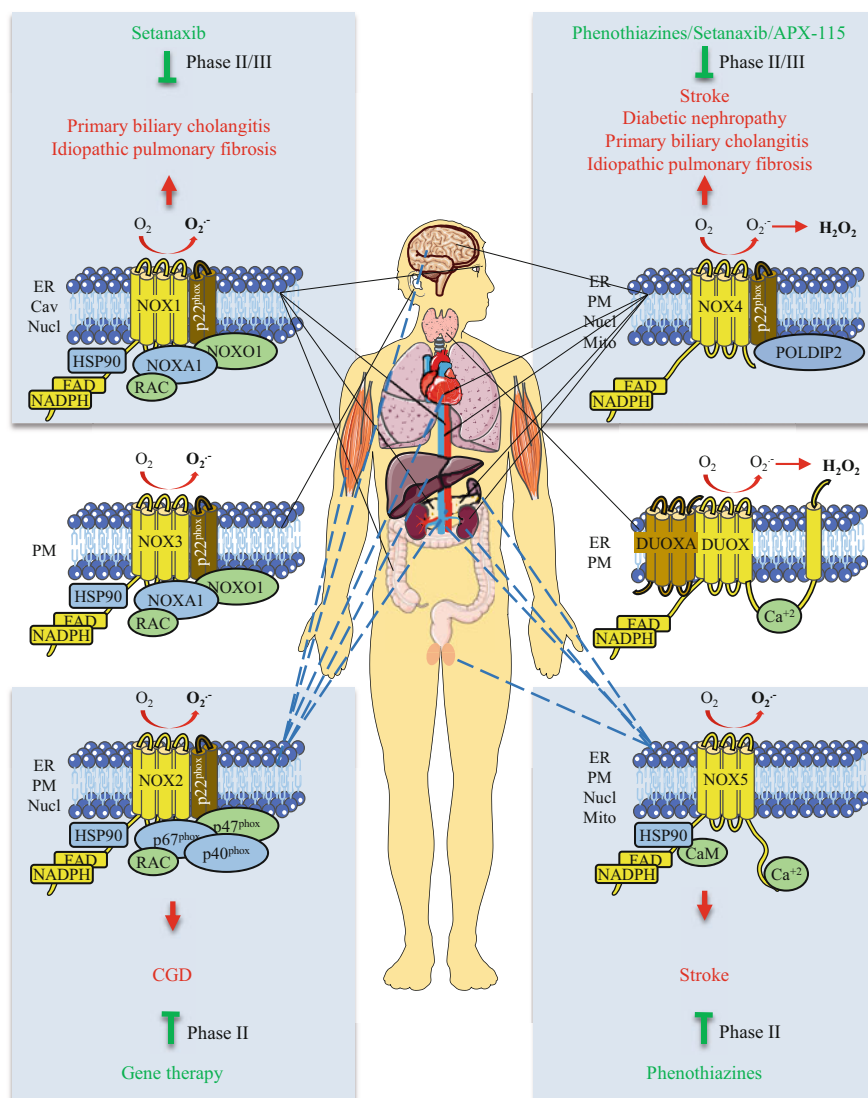


Fig. 1 NADPH oxidases and their clinical relevance. Seven NADPH oxidases, NOX1–5 and DUOX1–2, exist and possess a catalytic transmembrane subunit, NOX, which allows for NADPH oxidation through a FAD/NADPH-binding domain. NOXs have different membrane or cytosolic binding partners (p22^{phox}, DUOXA, NOXA1, NOXO1, RAC, HSP90, p67^{phox}, p40^{phox}, p47^{phox}, POLIDIP2) that are required for the enzymatic activity. NOXs are expressed in many organs: NOX1 in the colon, blood vessels, retina, and pancreas; NOX2 in the blood vessels, neurons, skeletal muscles, liver, and heart; NOX3 in the inner ear; NOX4 in the neurons, heart, blood vessels, kidney, and pancreas; NOX5 in the spleen, testis, kidney, and blood vessels; and DUOXs in the thyroid gland. NOXs are also expressed in different subcellular locations including endoplasmic reticulum (ER), caveolae (Cav), nucleus (Nucl), plasma membrane (PM), and mitochondria (Mito). The therapeutically relevant NOXs (in light blue boxes) include NOX1 and NOX4 being tested in

Matsushima et al. 2013; Perrotta et al. 2011; Van Buul et al. 2005; Wu et al. 2010; Yu et al. 2014) and DUOX1–2 at the apical membrane and endoplasmic reticulum (De Deken et al. 2000, 2002; Schwarzer et al. 2004).

Given all these structural characteristics, dissimilar tissue distribution, and sub-cellular localization of the NOX family members, they show distinct modes of activation (reviewed in (Brandes et al. 2014)) except NOX4 (Zhang et al. 2019) and the DUOXs (Aliasgharzadeh et al. 2019; Azmoonfar et al. 2018; Farhood et al. 2019) which are constitutively active and regulated at the expressional level. Additionally, NOXs differ in their ROS product, i.e., NOX1–3 and NOX5 produce superoxide, while NOX4 and DUOX1–2 produce hydrogen peroxide (Altenhofer et al. 2015). Overall, full characterization of the NOX enzymes is still deficient, yet by generating reliable high-quality antibodies and isoform-specific NOX inhibitors, it might be achievable.

2 NADPH Oxidases in Physiology

Being the sole and primary function of the NOX enzymes, ROS production should not be viewed mainly as disease trigger and metabolic waste. Indeed, ROS from NOXs among others contribute to several physiological functions such as host defense, angiogenesis, cell survival, tissue regeneration, hearing, hormone synthesis and sensitivity, vasodilation, and cell signaling (Elbatreek et al. 2019; Jiang et al. 2011). These functions need to be taken also into consideration as potential sources of side effects when NOX inhibitors are used therapeutically.

With respect to individual NOXs, NOX1-derived ROS slow down apoptosis of gastric mucosal cells and thereby regulate their growth (Teshima et al. 2000). In the colon, NOX1 is a part of the innate immune response, promotes cell proliferation and differentiation, stimulates mucosal wound repair, and prevents inflammation (Coant et al. 2010; Geiszt et al. 2003a; Kajla et al. 2012; Kato et al. 2016; Moll et al. 2018; Rokutan et al. 2006). Moreover, NOX1 plays a role in cell signaling via inhibiting protein tyrosine phosphatases and thus inactivation of peroxiredoxin 1, an enzyme that metabolizes/detoxifies hydrogen peroxide, thereby allowing the localized and transient accumulation of hydrogen peroxide for cell signaling (Woo et al. 2010). In the brain, NOX1 is suggested to suppress neuronal differentiation via inhibiting excessive neurite outgrowth (Ibi et al. 2006).

NOX2 is a key player in the innate host defense against infection. Mutations in genes encoding components of the NOX2 enzyme complex lead to chronic

Fig. 1 (continued) primary biliary cholangitis and idiopathic pulmonary fibrosis, NOX4 also in diabetic nephropathy and stroke, NOX2 in chronic granulomatous disease (CGD), and NOX5 in stroke. NOX inhibitors including setanaxib, APX-115, and phenothiazines and NOX2 gene therapy are being tested in Phase II/III clinical trials for these indications. Abbreviations: CaM, calmodulin; HSP90, heat shock protein 90; NOXA1, NADPH oxidase activator 1; NOXO1, NADPH oxidase organizer 1; POLDIP2, polymerase δ -interacting protein 2

granulomatous disease (CGD) which is characterized by immunodeficiency and recurrent and life-threatening infections (Panday et al. 2015). ROS from NOX2 can kill the attacking microorganisms directly by oxidative damage of proteins, lipids, and DNA and indirectly by activation of downstream signaling (Iles and Forman 2002). In addition to host defense, NOX2 might be involved in learning and memory as CGD patients show cognitive deficits and NOX2 mutant mice have mild memory impairment (Kishida et al. 2006; Pao et al. 2004). NOX2 also might have a protective function against colon inflammation as CGD patients also exhibit non-infective colitis (Pao et al. 2004). Moreover, NOX2 mediates the renal vasoconstriction effect of angiotensin and thus regulates the normal renal blood flow (Haque and Majid 2004) and enhances skeletal muscle metabolism and insulin sensitivity (Henriquez-Olguin et al. 2019). Apart from NOX2, the key physiological roles of NOX3 are mainly known in the inner ear. Mutation in the *NOX3* gene results in a lack of otoconia formation and vestibular dysfunction as shown in “head-tilt” mutant mice (Paffenholz et al. 2004). Also, recently, NOX3, together with NOX5, has been suggested to induce differentiation of human oligodendrocytes (Accetta et al. 2016).

NOX4 has a plethora of physiological and protective roles. This is probably explained by its constitutive activity, wide distribution, and production of hydrogen peroxide which is an omnipresent signaling molecule (Elbatreek et al. 2019; Guo and Chen 2015; Veal and Day 2011; Zhang et al. 2019). However, knocking out the *NOX4* gene in mice and rats does not result in an obvious phenotype or affect the life span of the animals (Kleinschnitz et al. 2010; Rezende et al. 2017). NOX4 enhances hormone-stimulated sodium and water transport in the kidney (Feraille et al. 2014; Lu et al. 2016), adipocytes differentiation (Schroder et al. 2009), insulin sensitivity in the liver and adipose tissue (Mahadev et al. 2004; Taniguchi et al. 2006), glucose-stimulated insulin secretion (Plecita-Hlavata et al. 2020), autophagy in cardiomyocytes (Kouroku et al. 2007), hippocampal neurogenesis, memory formation (Choi et al. 2019; Yoshikawa et al. 2019), angiogenesis, and vasodilation (Burgoyne et al. 2007; Drummond et al. 2000). NOX4 also activates downstream redox-sensitive proteins that play important roles in cell proliferation, migration, and apoptosis (Guo and Chen 2015). Further, NOX4 protects the vasculature from ischemic and inflammatory stress such as in atherosclerosis (Gray et al. 2016; Schroder et al. 2012).

NOX5 is the least studied NOX, and its physiological roles are not fully understood due to its absence in rodents. However, it has been suggested to regulate cell signaling and function (Fulton 2009) and contribute to sperm motility and viability (Ghanbari et al. 2018), as well as vascular smooth muscle cells contraction (Montezano et al. 2018). DUOX enzymes appear to be important for thyroid hormone synthesis. Mutations in the *DUOX2* lead to disruption of thyroid hormone synthesis and hypothyroidism (Moreno et al. 2002). DUOXs also play a role in host defense in the gastrointestinal tract and lung epithelia (van der Vliet et al. 2018).

Most of the abovementioned functions of NOXs derive from preclinical data and the physiological roles of NOXs in humans remain poorly understood. While the

biological effects of NOXs are important for health, dysfunctions in these enzymes may lead to pathology.

3 NADPH Oxidases in Pathology

Several pathophysiological roles have been validated for NOX enzymes, and thus several diseases are largely based on NOX dysregulation (Casas et al. 2015; Dao et al. 2015) (Fig. 1).

3.1 NOX1

NOX1 is involved in fibrotic diseases in many organs (Kato and Hecker 2020). Current clinical studies to target NOX1, together with NOX4, are focused on idiopathic pulmonary fibrosis and primary biliary cholangitis (a fibrotic orphan disease) (Table 1). Moreover, NOX1 seems a clinically relevant target in GI disorders. On the one hand, defects in *NOX1* are found in patients with very-early-onset inflammatory bowel diseases (Hayes et al. 2015; Scherz-Shouval and Elazar 2011). Indeed, some variants in *NOX1* are associated with complete loss of function of the gene product and with loss of ROS production in IBD patients (Schwerd et al. 2018). On the other hand, NOX1 overexpression is associated with colon and gall bladder cancers (Wang et al. 2019; Juhasz et al. 2017; Laurent et al. 2008). Besides GI-related disorders, diabetic vascular complications including diabetes-accelerated atherosclerosis (Gray et al. 2013) and retinopathy (Wilkinson-Berka et al. 2014) are potential conditions for clinical testing of NOX1 inhibitors.

3.2 NOX2

NOX2 genetic defects or inhibition are associated with immune deficiency and increased risk of infection, particularly in diabetes (Gray et al. 2013). Mutations in *CYBB*, *CYBA*, *NCF1*, *NCF2*, and *NCF4* genes, encoding NOX2, p22phox, p47phox, p67phox, and p40phox, respectively, cause CGD. Around 70% of CGD cases are due to mutations in *CYBB* (called X-linked CGD) resulting in decreased NOX2 expression, activity, or both. Therefore, CGD leads to immunodeficiency and increases susceptibility to recurrent and life-threatening infections due to fungal or bacterial pathogens (Giardino et al. 2017; O'Neill et al. 2015). As a key enzyme of the innate and inflammatory response, NOX2 has been suggested to be involved in an excessive and unlikely number of disease models (Casas et al. 2015; Elbatreek et al. 2019) which might indicate a possible positive publication bias, as shown by a meta-analysis of NOX2 studies in stroke (Kleikers et al. 2015), or an epiphenomenon without therapeutic relevance.

Table 1 NOX inhibitors and gene therapy for CGD and their clinical status

	Isoform	Indication	Clinical trial	Results/status
<i>NOX inhibitors</i>				
GKT137831 (GKT-831 or setanaxib)	1,4	Type 2 diabetes mellitus nephropathy	Phase I (NCT03740217) A double-blind, placebo-controlled, randomized, multicenter, parallel group, Phase II (NCT02010242)	Safe Reduced several secondary efficacy endpoints. However, improvements in albuminuria, the study's primary efficacy endpoint, was not achieved after 12 weeks of treatment
		Type 1 diabetes mellitus nephropathy	A double-blind, placebo-controlled, randomized, multicenter, with two parallel arms Phase II (U1111-1187-2609)	Ongoing in Australia and expanded to Europe and New Zealand
		Primary biliary cholangitis/ cirrhosis (PBC)	A double-blind, placebo-controlled, randomized, multicenter, parallel group, Phase II (NCT03226067)	Achieved rapid, dose- and time-dependent reductions in markers of cholestatic bile duct and liver injury. These reductions in disease activity were highly significant for both ALP and GGT
		Idiopathic pulmonary fibrosis (IPF)	A double-blind, placebo-controlled, randomized, multicenter, parallel group, Phase II (NCT03865927)	Not yet recruiting
APX-115	1,2,4	Type 1 diabetes mellitus nephropathy	Phase II (10.4062/ biomolther.2019.188)	Not yet recruiting
Perphenazine	4,5	Stroke	Phase II, repo-stroke (repo-trial.Eu) (EduraCT no. 2019–000474-31)	Not yet recruiting
<i>Gene therapy</i>				
Lentiviral gene therapy	2	X-linked chronic granulomatous disease (X-CGD)	Phase I/II, non-randomized, multicenter, open-label (NCT02234934 and NCT01855685)	The primary objective (to assess the safety and evaluate the efficacy and stability of biochemical and functional reconstitution in the

(continued)

Table 1 (continued)

	Isoform	Indication	Clinical trial	Results/status
Retroviral gene therapy				progeny of engrafted cells at 12 months) was met in six of the nine patients
			Phase I/II, non-randomized, open-label (NCT03645486)	Recruiting
			Phase I/II, non-randomized, monocentric open-label (NCT02757911)	Recruiting
			Phase I/II, non-randomized, single center, uncontrolled, open-label (NCT00778882)	In progress
			Phase I/II, non-randomized, open-label (NCT01906541)	Recruiting

3.3 NOX3

Being expressed in the inner ear, NOX3 appears to be a key target for hearing loss. Preclinical data show that noise exposure causes overexpression of NOX3 that results in cochlear inflammation, apoptosis, and eventually hearing loss (Dhukhwa et al. 2019). NOX3 also was shown to be associated with drug-induced hearing loss (Rybak et al. 2012). Genetic clinical studies show that NOX3 is associated with noise-induced hearing loss (Zhao et al. 2020) and pulmonary hypertension as shown in a recent GWAS (Yin et al. 2018). Further clinical and therapeutic validation of NOX3 in these conditions needs to be investigated.

3.4 NOX4

Preclinical data suggest that NOX4 is involved in many diseases including diabetic kidney disease (Jha et al. 2014, 2016), cancer (Lin et al. 2017), fibrosis of the liver (Lan et al. 2015) and lung (Carnesecchi et al. 2011), and ischemic stroke (Casas et al. 2017, 2019a; Kleinschnitz et al. 2010). Genetic clinical data shows that NOX4 is associated with an increased risk of stroke (He et al. 2018). Diversely, the role of NOX4 in cardiovascular disorders such as hypertension and atherosclerosis is likely limited. Indeed NOX4 seems protective in diabetes-accelerated atherosclerosis (Gray et al. 2016; Schurmann et al. 2015) and myocardial infarction-induced cardiac remodeling (Mongue-Din et al. 2017). Clinical studies on NOX4 are focused on

stroke for acute indications and diabetic kidney disease and fibrotic conditions for chronic indications. Yet, due to the dual effects of NOX4 and its plentiful biological functions in many organs, chronic NOX4 inhibition seems a less attractive approach. In cancer, however, targeting NOX4 needs to be examined given its metabolic, anti-apoptotic, and pro-angiogenic properties.

3.5 NOX5

NOX5 appears as a promising target in cardiovascular diseases, i.e., hypertension and atherosclerosis (Guzik et al. 2008; Touyz et al. 2019). Our recent findings show that NOX5 levels in endothelial microparticles are increased in a subgroup of hypertensive patients leading to eNOS uncoupling and endothelial dysfunction. NOX5 might also be a potential target in stroke (Casas et al. 2019b), myocardial infarction (Hahn et al. 2012), cancer (Antony et al. 2017), diabetic nephropathy (Jha et al. 2017b), aortic aneurysm (Guzik et al. 2013), and hemorrhagic transformation (Won et al. 2011).

3.6 DUOXs

The clinical relevance of targeting DUOX isoforms is not yet clear. Preclinical evidence suggests that DUOXs might contribute to immune and allergic disorders (van der Vliet et al. 2018) and can be targeted for radiation-induced thyroid cancer (Ameziane-El-Hassani et al. 2015). Similar to *NOX1*, mutations in *DUOX2* are found in patients with very-early-onset inflammatory bowel diseases (Hayes et al. 2015).

Taken together, given the diverse effects of the NOX enzymes both in physiology and disease, benefit-risk assessments should be considered as exemplified by NOX2 inhibition which is associated with immunodeficiency and infection (Gray et al. 2013; Panday et al. 2015). Also, inhibition of DUOX2 can result in hypothyroidism and bowel inflammation (Hayes et al. 2015). Similarly, inhibiting NOX1 might enhance gut inflammation (Schwerdt et al. 2018), and inhibiting NOX4 might promote atherosclerosis (Gray et al. 2016) and enhance the risk of kidney fibrosis (Nlandu Khodo et al. 2012) and liver cancer (Crosas-Molist et al. 2017). Acute indications such as ischemic stroke are likely to have, however, a low risk-benefit profile.

4 NADPH Oxidases Inhibitors

Despite the fact that NOX inhibitors are already in the clinic, the field has still to be considered relatively immature. There are no compounds available that deserve the term NOS isoform specific. Most compounds are pan-NOX inhibitors. Two recent analyses identified compounds with some isoform preference (Augsburger et al.

2019; Dao et al. 2019), and it has been shown that by using a panel of marginally selective inhibitors, specific isoforms, such as NOX4, could be validated pharmacologically (Dao et al. 2019). Considering the critical roles of NOXs in the pathogenesis of many diseases, they have been suggested as promising therapeutic targets. Several small molecules have been thought to inhibit NOX activity; however, majority were unspecific due to off-target effects. These molecules include, for example, diphenyleneiodonium (DPI) and apocynin. The former is a flavoprotein inhibitor and thus inhibits many other enzymes besides NOXs, while the latter has non-specific ROS scavenging properties (Altenhofer et al. 2015). Likewise, some other recently developed NOX inhibitors are unspecific such as VAS2870, ML-171, and GKT136901 (Augsburger et al. 2019; Dao et al. 2019). Only a few compounds are claimed to be specific NOX inhibitor in preclinical studies including GSK2795039 which selectively inhibits NOX2 (Hirano et al. 2015), GLX7013114 for NOX4 (Wang et al. 2018), and Ewha-18278 (APX-115) for NOX1, NOX2, and NOX4 (Cha et al. 2017).

NOX inhibitors currently being tested in the clinical phase are focused on fibrotic and neurovascular disease indications with NOX1, 4, and 5 as the main isoforms to be targeted. GKT137831 (setanaxib or GKT-831) claimed as a NOX1/4 dual inhibitor and a partial Nox5 inhibitor is the first-in-class NOX inhibitor to reach the clinical trial stage (Table 1). Setanaxib was safe and showed encouraging pharmacokinetic properties during Phase I study. Subsequently, it was tested in Phase II clinical trial for nephropathy in type 2 diabetes patients, yet the primary efficacy endpoint, i.e., albuminuria reduction, was not achieved. However, several other secondary efficacy endpoints were reached such as maximal inhibition of the renin-angiotensin-aldosterone system. In another Phase II trial focused on primary biliary cholangitis, setanaxib has succeeded and met its primary and secondary efficacy endpoints. Two additional Phase II clinical trials using setanaxib are ongoing, for idiopathic pulmonary fibrosis and kidney disease in type 1 diabetes. The second NOX inhibitor to reach the clinical trial stage is APX-115 which is moving from Phase I to II for diabetic kidney disease (Lee et al. 2020). Phenothiazines, already marketed for some indications, i.e., antipsychotic, show pan NOX inhibition activity in some preclinical studies (Seredenina et al. 2015, 2016). One clinical trial is planned to repurpose phenothiazines into stroke (Repo-Stroke).

Based on preclinical data, further indications for NOX inhibitors might also have potential toward the clinical application. For example, in ischemic retina disease and diabetic retinopathy, setanaxib and its analogue GKT136901 showed favorable effects (Appukuttan et al. 2018; Jiao et al. 2019; J L Wilkinson-Berka et al. 2013). Also, in cardiovascular disorders, including diabetes-associated atherosclerosis and hypertensive cardiac remodeling and hypertrophy, and liver fibrosis, setanaxib attenuated inflammatory and fibrotic markers (Gray et al. 2013; Sun et al. 2017; Zeng et al. 2019; Zhao et al. 2015) even when the treatment was delayed (Gray et al. 2017). VAS2870 which is a pan NOX inhibitor showed vascular protective effects in pulmonary hypertension (Li et al. 2019) and Alzheimer's disease (Abubaker et al. 2019). ML090 which has preferential activity toward NOX5 was beneficial in stroke

(Casas et al. 2019b; Dao et al. 2019). Collectively, NOX inhibition seems a promising therapeutic strategy with a broad range of clinical applications and warrants further investigations.

5 Advanced Therapies

Currently, the only known cure for CGD is allogeneic hematopoietic stem cell transplantation which is a high-risk procedure and associated with severe disability or death (Kang et al. 2011b). Only one drug is approved to treat/manage CGD, interferon gamma-1b that reduces the frequency and severity of serious infections associated with the disease (Miller et al. 2009). Current clinical research suggests that gene therapy holds great promise in curing CGD obviating the need for a transplantation donor and eliminating the risks associated with stem cell transplantation (Keller et al. 2018). What also makes gene therapy an attractive treatment for CGD is that restoration of normal NOX activity in only 10–20% of circulating neutrophils is sufficient to achieve significant clinical benefit (Keller et al. 2018). Early clinical trials on gene therapy for CGD were mainly based on γ -retroviral vectors that can only infect mitotically active cell types (Escors and Breckpot 2010). These studies failed to show efficacy and were associated with insertional mutagenesis, due to upregulation of proto-oncogene expression (Kang et al. 2011a; Keller et al. 2018). To overcome the latter issue, self-inactivating (SIN) retroviral vectors have been developed (Thornhill et al. 2008) and are being tested in clinical trials (NCT01906541). More recent clinical trials are using SIN lentiviral vectors (complex retroviruses), which unlike γ -retroviral vectors are capable of transducing quiescent cells and devoid of insertional toxicities (Escors and Breckpot 2010). The preliminary results from these lentiviral gene therapy trials (NCT02234934 and NCT01855685) are encouraging (Kohn et al. 2020) (Table 1).

6 Conclusions

NOX enzymes are primary sources of ROS, and their activation results in the activation of secondary ROS sources, i.e., ROS-dependent ROS production or the kindling-bonfire sequence. These secondary ROS sources include uncoupled nitric oxide synthase (NOS), xanthine oxidase, and dysfunctional mitochondria (Zhang et al. 2019). Therefore, NOX inhibition might represent an intelligent therapeutic strategy in ROS-related diseases as it targets the origin. However, none of the ROS sources act on their own, and different ROS forming enzymes will affect different targets. Thus, combinations are most likely more effective than single target strategies, which may lead to better efficacy and reduced side effects. As NOX inhibitors have entered clinical trials, two main aspects should be considered, specificity and isoform selectivity. Most of the NOX inhibitors in development are non-specific even the most advanced ones, setanaxib and GKT136901, have ROS scavenging activities (Augsburger et al. 2019; Dao et al. 2019). Isoform selectivity

of the NOX inhibitors is also important given the physiological tissue- and cell-specific effects of NOXs. Applying a NOX inhibitor panel approach could be an option for NOX target validation (Dao et al. 2019). Further lead optimization of the current NOX inhibitors might help find isoform-selective compounds. Finally, ROS have important beneficial signaling functions. Thus, acute interventions such as in stroke (NOX4 and NOX5) appear safer than chronic therapies suppressing NOX1 or NOX4. Clinical trials in both directions are under way (NCT03865927, EudraCT No. 2019-000474-31) and will answer this by the early 2020s.

Acknowledgments This review project has received funding from the European Union's Horizon 2020 research and innovation program under grant agreement No 777111 (REPO-TRIAL). This reflects only the author's view, and the European Commission is not responsible for any use that may be made of the information it contains.

Conflict of Interest None

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